

# Signaling Inositol Polyphosphate-5-phosphatase

## CHARACTERIZATION OF ACTIVITY AND EFFECT OF GRB2 ASSOCIATION\*

(Received for publication, November 25, 1996)

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An inositol polyphosphate-5-phosphatase (SIP-110) that binds the SH3 domains of the adaptor protein GRB2 was produced in Sf9 cells and characterized. SIP-110 binds to GRB2 *in vitro* with a stoichiometry of 1 mol of GRB2/0.7 mol of SIP-110. GRB2 binding does not affect enzyme activity implying that GRB2 serves mainly to localize SIP-110 within cells. SIP-110 hydrolyses inositol (Ins)(1,3,4,5)P<sub>4</sub> to Ins(1,3,4)P<sub>3</sub>. The enzyme does not hydrolyze Ins(1,4,5)P<sub>3</sub> that is a substrate for previously described 5-phosphatases nor does it hydrolyze phosphatidylinositol (PtdIns)(4,5)P<sub>2</sub>. SIP-110 also hydrolyzed PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> as did recombinant forms of two other 5-phosphatases designated as inositol polyphosphate-5-phosphatase II, and OCRL (the protein that is mutated in oculocerebrorenal syndrome). The inositol polyphosphate-5-phosphatase enzyme family now is represented by at least 9 distinct genes and includes enzymes that fall into 4 subfamilies based on their activities toward various 5-phosphatase substrates.

The phosphatidylinositol signaling pathway serves as the signaling mechanism for various extracellular agonists that stimulate calcium ion mobilization, protein phosphorylation, and cell proliferation (1, 2). In response to receptor stimulation, phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>)<sup>1</sup> to generate Ins(1,4,5)P<sub>3</sub> and diacylglycerol. Ins(1,4,5)P<sub>3</sub> is further converted to Ins(1,3,4,5)P<sub>4</sub> by the action of a 3-kinase. These two soluble inositol phosphates are involved in calcium ion mobilization, and the latter has recently also been suggested to play a role in regulation of a Ras GAP1 (3). Specific 5-phosphatase enzymes hydrolyze Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> by converting them to Ins(1,4)P<sub>2</sub> and Ins(1,3,4)P<sub>3</sub>, respectively. These products are inactive in calcium mobilization (1, 2). The phosphatidylinositols PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> also serve signaling functions (4). These lipids may regulate cellular secretion (5, 6) and actin assembly (7–9), and they bind various proteins containing protein tyrosine binding, SH2, and pleckstrin homology domains (10–12). PtdIns(3,4,5)P<sub>3</sub> is absent or maintained at very low

levels in most cells except following stimulation with agonists that activate the PtdIns 3-kinase (13–17). Since PtdIns(3,4,5)P<sub>3</sub> is produced after PtdIns 3-kinase activation and is not a substrate for phospholipase C enzymes, it is suggested to act as a second messenger, possibly by activating a serine/threonine kinase, c-Akt (14, 18, 19).

There are now at least 9 distinct genes for 5-phosphatases or proteins with conserved 5-phosphatase sequences. The most recently identified of these are the 110-kDa SIP-110 (20) and the 133-kDa SHIP or SIP-130 (20–24). cDNAs encoding these proteins were cloned from human and mouse cDNA libraries, respectively, based on their ability to associate with the adaptor protein GRB2 (20–22) or their homology to 5-phosphatases or their ability to form complexes with the immunoreceptor-based tyrosine activation motif of mast cells (23, 24). The predicted amino acid sequences of the human 110-kDa protein and the 133-kDa mouse form are 86% identical over 969 amino acids, and peptide sequence from the mouse 133-kDa protein suggests that they are alternatively spliced products of a single gene (20, 21). Both associate with the SH3 domains of GRB2 through proline-rich sequences in their C-terminal portion. In addition, the 133-kDa protein contains an N-terminal SH2 domain that the 110-kDa protein lacks, becomes phosphorylated on tyrosine, and associates with the tyrosine-phosphorylated adaptor protein Shc (20–22). These 5-phosphatases hydrolyze only the 3-phosphate-containing inositol phosphates, Ins(1,3,4,5)P<sub>4</sub> and PtdIns(3,4,5)P<sub>3</sub> (20, 21, 23). We now report the enzyme activity and products of recombinant 110-kDa SIP-110 and the effects of GRB2 on its activity.

### EXPERIMENTAL PROCEDURES

**Materials**—All <sup>3</sup>H-labeled inositol phosphate isomers and [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> were purchased from DuPont NEN. PtdIns(4,5)P<sub>2</sub> and anti-HA antibody 12CA5 were purchased from Boehringer Mannheim. All unlabeled inositol phosphate isomers were purchased from Boehringer Mannheim or Calbiochem. Horseradish peroxidase-linked anti-mouse IgG was purchased from Bio-Rad. ECL Western blotting detection reagents were purchased from Amersham Life Sciences, and the 9E10 anti-Myc antibody was from Oncogene Science. Silica Gel 60 TLC plates (20 × 20 cm, 0.2 mm) were from Merck. The Adsorbosphere SAX HPLC column was purchased from Alltech; the Partisil 10 SAX HPLC column was purchased from Whatman.

**Expression of Recombinant Proteins**—The complete coding sequence of SIP-110 (20) was expressed in Sf9 insect cells as a GST fusion protein with an intermediate HA tag using a baculovirus expression vector pVIKS as described (25). Human 5-phosphatase I was expressed as a 412-amino acid protein (26, 27) using the pVL1392 baculoviral expression vector and BaculoGold transfection kit from PharMingen. Human 5-phosphatase II used in these studies was 5PtaseS consisting of amino acids 250–942 of the predicted amino acid sequence (28). An N-terminal truncated version of OCRL was expressed in Sf9 cells as described (29). Native human GRB2, GRB2 dbm, and GRB3.3 with a C-terminal Myc tag were expressed as GST fusion proteins in Sf9 cells using pVIKS (25). GRB2 dbm contains mutations (P49L and E203R) in the N- and C-terminal SH3 domains that are the human counterparts of *Caenorhabditis elegans*

\* This work was supported by Grants HL14147 (Specialized Center for Research in Thrombosis) and HL16634 and HL32898 from the National Institutes of Health and Chiron Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: PtdIns, phosphatidylinositol; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; IP, inositol phosphate; OCRL, oculocerebrorenal syndrome.

Sem-5 mutations (30), and GRB3.3 contains an SH2 domain deletion (31). Mutations were generated according to Higuchi (32). Human GRB2 was expressed as a GST fusion protein in *Escherichia coli* using pGexKT (33).

**Baculovirus Expression and Enzyme Activity**—Sf9 insect cells were grown in TNM-FH medium with 10% heat-inactivated fetal calf serum and 100 µg of gentamicin/ml. Approximately  $3 \times 10^6$  insect cells were infected with baculovirus encoding either a protein tyrosine phosphatase MEG-01 that served as a "negative control" (34), a 5-phosphatase, or native or mutant GRB2. For assays of enzyme activity, the cells were harvested from 60-mm dishes 3 days after infection and sonicated in 300 µl of Tris, pH 7.5 (20 mM), NaCl (150 mM), MgCl<sub>2</sub> (3 mM), EGTA (2 mM), β-mercaptoethanol (10 mM), phenylmethylsulfonyl fluoride (1 mM), benzamidine (10 µg/ml), pepstatin A (1 µM), and leupeptin (10 µg/ml). Sonicates were centrifuged at 16,000 × g for 10 min and supernatants were tested for enzyme activity. SIP-110 was purified on glutathione-agarose and eluted with glutathione for use in determining  $K_m$  and  $V_{max}$  for InsP<sub>4</sub> hydrolysis and in heat inactivation experiments.

Assay of 5-phosphatase activity using [<sup>32</sup>P]Ins(1,4,5)P<sub>3</sub>, [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub>, and [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> was as described (35–37). [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> was prepared as described (38) using vesicles composed of 0.5 mg of PtdIns(4,5)P<sub>2</sub> and 0.5 mg of phosphatidylserine and recombinant PtdIns 3-kinase (39) immunoprecipitated from COS cell lysates with antibody directed against an HA tag in 1 ml. TLC purified [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> was evaporated under N<sub>2</sub> with phosphatidylserine and resuspended into vesicles with approximately 0.5 mg of phosphatidylserine/ml. Reaction mixtures (25 µl) contained the indicated amounts of Sf9 supernatant in Tris, pH 7.5 (50 µM), MgCl<sub>2</sub> (10 mM), and 600–1500 cpm of [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> in vesicles with 2.5 µg of phosphatidylserine. Reaction mixtures were incubated at 37 °C for up to 30 min. Hydrolysis was determined as described (38).

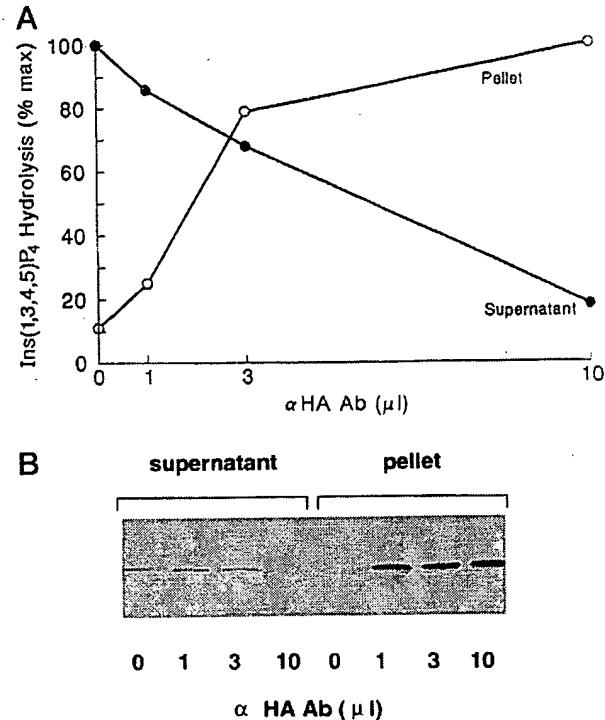
**Proof of the Product of Hydrolysis of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> by SIP-110**—The products formed from incubation of SIP-110 with [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> were separated by HPLC on an Adsorbosphere SAX column equilibrated in 20 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> at pH 3.5. Products were eluted with a linear gradient from 0 to 0.75 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> over 100 min. The products formed from incubation of SIP-110 product with inositol polyphosphate-1-phosphatase (40) or inositol polyphosphate-4-phosphatase (41) were separated by HPLC on a Partisil 10 SAX column equilibrated in 40 mM ammonium formate/formic acid, pH 3.5. Products were eluted with a linear gradient from 0 to 0.75 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> over 100 min. The products formed from incubation of SIP-110 product with inositol polyphosphate-1-phosphatase (40) or inositol polyphosphate-4-phosphatase (41) were separated by HPLC on a Partisil 10 SAX column equilibrated in 40 mM ammonium formate/formic acid, pH 3.5. Products were eluted with a linear gradient from 0 to 0.75 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> over 100 min. The products formed from incubation of SIP-110 product with inositol polyphosphate-1-phosphatase (40) or inositol polyphosphate-4-phosphatase (41) were separated by HPLC on a Partisil 10 SAX column equilibrated in 40 mM ammonium formate/formic acid, pH 3.5. Products were eluted with a linear gradient from 0 to 0.75 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> over 100 min. One-ml fractions were collected, and radioactivity was measured in a liquid scintillation counter.

**Western Blotting**—All immunoblotting was done with anti-HA antibody used at 120 ng/ml. Secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG from Bio-Rad (1:5000 dilution), and blots were developed using ECL (Amersham).

**GRB2 Association**—For experiments determining the association of SIP-110 with GRB2, Sf9 cells expressing SIP-110, native GRB2, GRB2 dbm, or GRB3.3 were lysed in Tris, pH 8 (20 mM), NaCl (140 mM), glycerol (10%), Triton X-100 (1%), MgCl<sub>2</sub> (3 mM), phenylmethylsulfonyl fluoride (1 mM), benzamidine (10 µg/ml), pepstatin A (1 µM), and leupeptin (10 µg/ml). Lysates were centrifuged at 16,000 × g for 10 min and the relative amount of recombinant protein in each lysate was determined by Western blotting with anti-HA antibody. Equimolar amounts of SIP-110 and GRB2 were mixed with either anti-Myc or anti-HA antibody in a 1:50 dilution and incubated at 4 °C overnight. Protein A-Sepharose (20 µl 50%) was added for 3 h and the protein A-Sepharose pellet was washed three times in the lysis buffer described above and three times in Tris, pH 7.5 (50 mM), and MgCl<sub>2</sub> (3 mM). Equal portions of each pellet were assayed for SIP-110 Ins(1,3,4,5)P<sub>4</sub> hydrolyzing activity or Western blotting with anti-HA antibody. For experiments using bacterial GRB2, GRB2 was purified on glutathione-agarose according to standard protocols and the amount of GRB2 was measured as absorbance at 280 nm. This GRB2 was mixed with SIP-110 from non-detergent-containing supernatants of Sf9 cells prepared as described for enzyme assays and assayed for hydrolysis of Ins(1,3,4,5)P<sub>4</sub> or PtdIns(3,4,5)P<sub>3</sub>.

## RESULTS

We expressed SIP-110 in Sf9 cells and assayed the soluble crude recombinant enzyme for the ability to hydrolyze soluble inositol polyphosphates. Hydrolysis of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> to an apparent InsP<sub>3</sub> product was Mg<sup>2+</sup> dependent while extracts from Sf9 cells expressing an irrelevant tyrosine phosphatase as a control did not hydrolyze this substrate. We confirmed that substrate hydrolysis in Sf9 supernatants expressing SIP-110

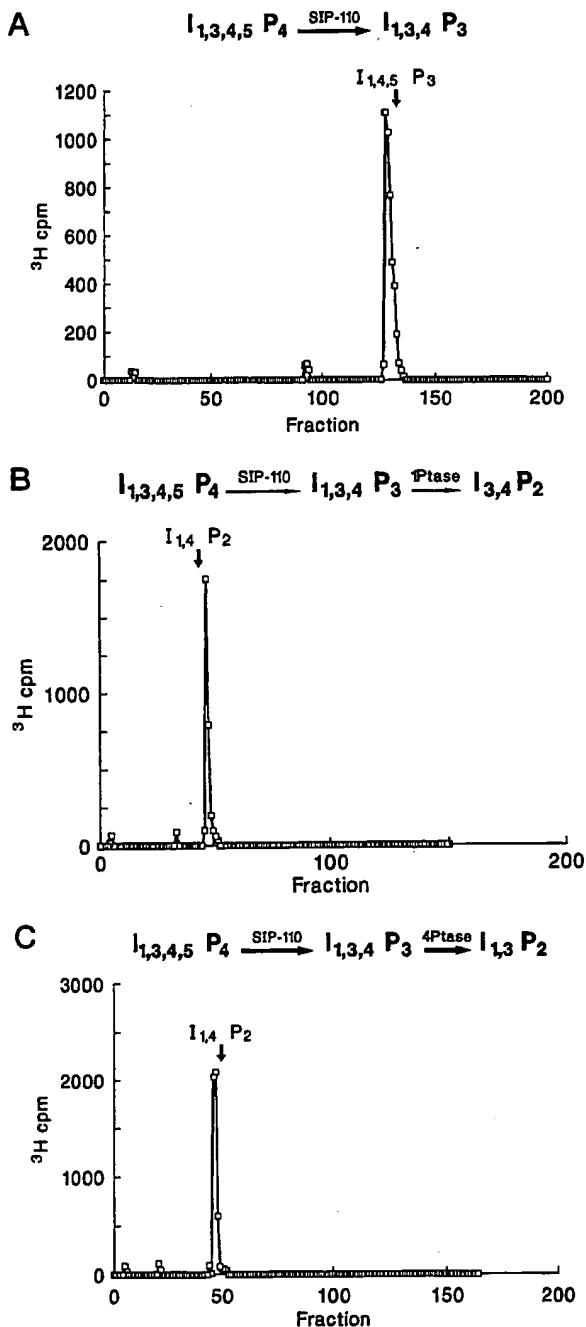


**Fig. 1. Immunoprecipitation of SIP-110.** Sf9 supernatant containing HA-tagged SIP-110 was incubated with a constant concentration of total IgG with an increasing amount of anti-HA antiserum overnight at 4 °C. IgG was bound to protein A-Sepharose, and the immune supernatant and the protein A-Sepharose pellet were assayed for ability to hydrolyze Ins(1,3,4,5)P<sub>4</sub> (A) or for Western blotting with anti-HA Ab (B).

was due to the SIP-110 by immunoprecipitating that protein with a monoclonal antibody to a hemagglutinin (HA) tag at the N terminus of the recombinant protein. Increasing amounts of antibody depleted InsP<sub>4</sub> hydrolyzing activity from Sf9 supernatants and resulted in appearance of activity in the protein A-Sepharose pellet (Fig. 1A). Amounts of antiserum required to immunoprecipitate InsP<sub>4</sub> hydrolyzing activity correlated with the amount of antiserum necessary to immunoprecipitate SIP-110 protein as determined by Western blotting with anti-HA antiserum (Fig. 1B).

We next determined the product of SIP-110 hydrolysis of Ins(1,3,4,5)P<sub>4</sub>. [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> was hydrolyzed to completion with recombinant SIP-110 and the reaction mixture was chromatographed by Adsorbosphere SAX HPLC. The product eluted as a single peak in the position of Ins(1,3,4)P<sub>3</sub>, slightly earlier than a [<sup>32</sup>P]Ins(1,4,5)P<sub>3</sub> internal standard (Fig. 2A). To confirm that the product of SIP-110 is Ins(1,3,4)P<sub>3</sub>, we further hydrolyzed this product with two other purified recombinant inositol polyphosphate phosphatases that remove specific phosphates from Ins(1,3,4)P<sub>3</sub>. Inositol polyphosphate-1-phosphatase further converted the SIP-110 product [<sup>3</sup>H]InsP<sub>3</sub> to an [<sup>3</sup>H]InsP<sub>2</sub> that eluted from Partisil 10 SAX HPLC slower than the [<sup>32</sup>P]Ins(1,4)P<sub>2</sub> internal standard in the position of Ins(3,4)P<sub>2</sub> (41) (Fig. 2B). Inositol polyphosphate-4-phosphatase converted the SIP-110 [<sup>3</sup>H]InsP<sub>3</sub> product to an [<sup>3</sup>H]InsP<sub>2</sub> that eluted from Partisil 10 SAX HPLC faster than the [<sup>32</sup>P]Ins(1,4)P<sub>2</sub> internal standard in the position of Ins(1,3)P<sub>2</sub> (42) (Fig. 2C). Thus SIP-110 is an inositol polyphosphate-5-phosphatase that converts Ins(1,3,4,5)P<sub>4</sub> to Ins(1,3,4)P<sub>3</sub>.

Of all soluble inositol polyphosphates tested as substrates for SIP-110, only Ins(1,3,4,5)P<sub>4</sub> was hydrolyzed (Table I). Most interestingly, there was no detectable hydrolysis of [<sup>32</sup>P]Ins(1,4,5)P<sub>3</sub> in numerous experiments performed under a wide



**FIG. 2.** Analysis of the product of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> hydrolysis by SIP-110. *A*, [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> (200 pmol) was incubated with SIP-110 (1 µg) for 1 h at 37 °C. A portion of this reaction mixture was quenched with 500 µl of cold water, mixed with 300 cpm of [<sup>32</sup>P]Ins(1,4,5)P<sub>3</sub> as an internal standard, and analyzed by Adsorbosphere SAX HPLC. Other portions of the SIP-110 reaction mixture described above were incubated with purified recombinant inositol polyphosphate-1-phosphatase (44) (*B*) or purified recombinant inositol polyphosphate-4-phosphatase (45) (*C*), quenched with 500 µl of cold water, mixed with 300 cpm of [<sup>32</sup>P]Ins(1,4)P<sub>2</sub> as an internal standard, and analyzed by Partisil 10 SAX HPLC.

range of pH conditions with a variety of salt and metal ion additions. This specificity for Ins(1,3,4,5)P<sub>4</sub> is distinct from the substrate specificity of other cloned or described 5-phosphatases that hydrolyze Ins(1,4,5)P<sub>3</sub> in addition to Ins(1,3,4,5)P<sub>4</sub>. A number of unlabeled inositol phosphate isomers were tested for their ability to serve as inhibitors of the hydrolysis of Ins(1,3,4,5)P<sub>4</sub> by SIP-110. Only two isomers, Ins(1,3,4)P<sub>3</sub> and Ins(1,5,6)P<sub>3</sub> gave greater inhibition than additional unlabeled

TABLE I  
Substrate specificity of SIP-110

|                              | Hydrolysis <sup>a</sup> | Inhibition <sup>b</sup> |
|------------------------------|-------------------------|-------------------------|
|                              | nmol/min/mg             | % activity remaining    |
| Ins(1,4)P <sub>2</sub>       | ND <sup>c</sup>         |                         |
| Ins(1,4,5)P <sub>3</sub>     | ND                      |                         |
| Ins(1,3,4)P <sub>3</sub>     | ND                      |                         |
| Ins(1,3,4,5)P <sub>4</sub>   | 100                     |                         |
| Ins(4)P                      |                         | 71                      |
| Ins(2,4)P <sub>2</sub>       |                         | 88                      |
| Ins(4,5)P <sub>2</sub>       |                         | 80                      |
| Ins(1,4)P <sub>2</sub>       |                         | 101                     |
| Ins(1,4,5)P <sub>3</sub>     |                         | 76                      |
| Ins(1,3,4)P <sub>3</sub>     |                         | 22                      |
| Ins(2,4,5)P <sub>3</sub>     |                         | 72                      |
| Ins(1,5,6)P <sub>3</sub>     |                         | 14 <sup>d</sup>         |
| Ins(1,4,5,6)P <sub>4</sub>   |                         | 48                      |
| Ins(3,4,5,6)P <sub>4</sub>   |                         | 48                      |
| Ins(1,3,4,5)P <sub>4</sub>   |                         | 49                      |
| Ins(1,2,5,6)P <sub>4</sub>   |                         | 59                      |
| Ins(1,3,4,5,6)P <sub>5</sub> |                         | 50                      |
| InsP <sub>6</sub>            |                         | 82                      |

<sup>a</sup> Assays were performed as described with 16–120 µM radiolabeled substrate for 30 min at 37 °C.

<sup>b</sup> Assays were performed with 8 µM [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> in the presence of 40 µM unlabeled competing inositol isomer.

<sup>c</sup> ND, none detected.

<sup>d</sup> Inhibitor present at only 35 µM final concentration.

Ins(1,3,4,5)P<sub>4</sub> substrate at the concentrations tested. Inhibition by Ins(1,3,4)P<sub>3</sub> most likely reflects product inhibition. Ins(1,5,6)P<sub>3</sub> was identified as a product of the metabolism of InsP<sub>5</sub> from avian erythrocytes (43). Its role in signaling in mammalian cells is unknown.

The substrate concentration dependence of hydrolysis of Ins(1,3,4,5)P<sub>4</sub> was measured as shown in Fig. 3. Purified SIP-110 hydrolyzed [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> to Ins(1,3,4)P<sub>3</sub> with a K<sub>m</sub> of 16 ± 2.1 µM (S.E., n = 4) and a V<sub>m</sub> of 9.3 ± 1.4 µmol/min/mg SIP-110 protein. The catalytic efficiency of this enzyme towards Ins(1,3,4,5)P<sub>2</sub> (V<sub>m</sub>/K<sub>m</sub>) is 0.58 indicating that it is the best enzyme in utilizing this substrate compared to the other 5-phosphatases studied to date (29).

A number of inositol polyphosphate-5-phosphatases have recently been shown to hydrolyze phosphatidylinositol phosphates. Consequently, we tested SIP-110 for its ability to hydrolyze PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. SIP-110 hydrolyzes [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> in a concentration (Fig. 4A) and time (data not shown) dependent manner. We detected no hydrolysis of [<sup>3</sup>H]PtdIns(4,5)P<sub>2</sub> under a variety of conditions with amounts of SIP-110 1000-fold greater than were necessary to detect hydrolysis of PtdIns(3,4,5)P<sub>3</sub> (Fig. 4A). We also tested three additional recombinant 5-phosphatases, 5-phosphatase I, 5-phosphatase II, and OCRL for their ability to hydrolyze [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub>. OCRL and 5-phosphatase II hydrolyze [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> to [<sup>32</sup>P]PtdInsP<sub>2</sub> (Fig. 4B). 5-Phosphatase I which does not hydrolyze PtdIns(4,5)P<sub>2</sub> (44) also failed to hydrolyze [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> even with 10-fold more enzyme than was necessary to detect hydrolysis by active 5-phosphatases (Fig. 4B). We compared the three active 5-phosphatases in their ability to hydrolyze [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub>. OCRL had the highest first-order rate constant (21.5 ± 4.5/min after 1 and 3 min of hydrolysis). 5-Phosphatase II and SIP-110 hydrolyzed [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> with first-order rate constants of 2.5 ± 1.0/min and 1.3 ± 0.5/min, respectively, after 1 and 3 min of hydrolysis. Thus, at least three 5-phosphatases can utilize PtdIns(3,4,5)P<sub>3</sub> as a substrate. Consistent with its preference for PtdIns(4,5)P<sub>2</sub> as a substrate when compared to Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub>, OCRL also rapidly hydrolyzes PtdIns(3,4,5)P<sub>3</sub>.

We confirmed that the SIP-110 protein was responsible for both the IP<sub>4</sub> and PtdIns(3,4,5)P<sub>3</sub> hydrolyzing activity by exam-

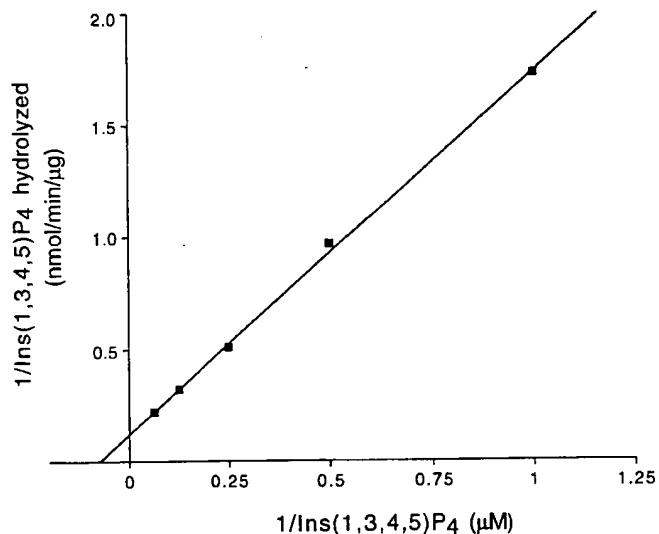


FIG. 3. Hydrolysis of  $\text{Ins}(1,3,4,5)\text{P}_4$  by SIP-110. Effect of varying the concentration of  $\text{Ins}(1,3,4,5)\text{P}_4$  on the rate of its hydrolysis by SIP-110. Purified SIP-110 (1.2 ng) incubated for 20 min at 37 °C with 1–16  $\mu\text{M}$  [ $^3\text{H}$ ] $\text{Ins}(1,3,4,5)\text{P}_4$ . Reaction products were separated by elution from Dowex AG 1-X8 with ammonium formate. These results are typical of four separate assays.

ining the heat inactivation of purified SIP-110 at 42 °C. The  $\text{IP}_4$  hydrolyzing activity and the  $\text{PtdInsP}_3$  hydrolyzing activity inactivate at essentially the same rate proving that the two activities reside in the same protein (Fig. 5). Further confirmation came from studies using SIP-110 with a mutation of aspartic acid 460 to alanine in the region of homology to other 5-phosphatases. A similar mutation in 5-phosphatase type II resulted in an inactive enzyme. SIP-110 D460A had no activity for either  $\text{InsP}_4$  or  $\text{PtdInsP}_3$  (Ref. 45, data not shown).

SIP-110 was initially cloned as a GRB2-binding protein. We next determined the stoichiometry of GRB2 binding to SIP-110 and whether the binding affected enzyme activity. In the experiment shown in Fig. 6, we mixed HA-tagged SIP-110 from Sf9 cell detergent lysates with equimolar amounts of native or mutant Myc-, HA-tagged GRB2. GRB2 dbm contains an inactivating single point mutation in each SH3 domain that inhibits association of SIP-110 in COS cells (19). GRB3.3 contains an inactivating SH2 domain deletion while retaining functional SH3 domains (31). We immunoprecipitated the GRB2 with antiserum to its Myc epitope and analyzed the association of SIP-110 with GRB2 by Western blotting with anti-HA antiserum or by assaying for [ $^3\text{H}$ ] $\text{Ins}(1,3,4,5)\text{P}_4$  hydrolysis. As shown in Fig. 6A, SIP-110 associates with native GRB2 in a stoichiometry of 1 GRB2:0.7 SIP-110 ( $0.7 \pm 0.1$ ,  $n = 5$ ), suggesting that the stoichiometry likely is 1:1. SIP-110 failed to bind in detectable amounts to GRB2 dbm confirming that the binding of SIP-110 is via the SH3 domains of GRB2. SIP-110 also binds to GRB3.3 although less well than to native GRB2 (ratio of GRB2 to SIP-110 is 12:1). We then assayed each immunoprecipitate for [ $^3\text{H}$ ] $\text{Ins}(1,3,4,5)\text{P}_4$  hydrolyzing activity. As shown in Fig. 6B, there was [ $^3\text{H}$ ] $\text{Ins}(1,3,4,5)\text{P}_4$  hydrolyzing activity in each GRB2 immunoprecipitate that contained SIP-110 protein. As a more accurate estimation of enzyme activity when associated with GRB2, we mixed SIP-110 with native GRB2 or GRB2 dbm as for immunoprecipitations above, but assayed SIP-110 activity without addition of antiserum or protein A-Sepharose. SIP-110 supernatant hydrolyzed  $5.5 \pm 1.8$  pmol of [ $^3\text{H}$ ] $\text{InsP}_4$ /min after incubation with equimolar amounts of native GRB2 supernatant and  $5.2 \pm 1.2$  pmol of [ $^3\text{H}$ ] $\text{InsP}_4$ /min after incubation with equimolar amounts of GRB2 dbm supernatant. Since these experiments were done

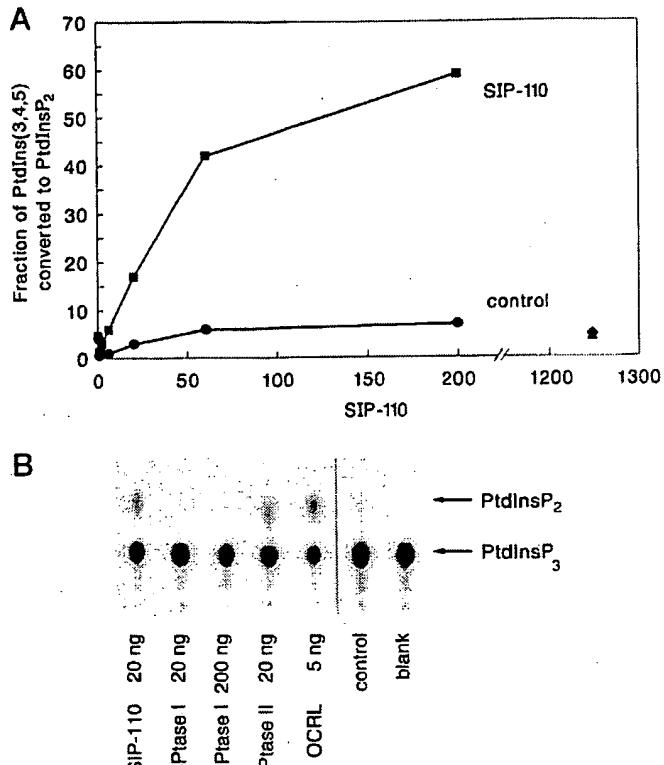


FIG. 4. Hydrolysis of  $\text{PtdIns}(3,4,5)\text{P}_3$  by SIP-110 and other 5-phosphatases. A, TLC purified [ $^{32}\text{P}$ ]PtdIns(3,4,5) $\text{P}_3$  (1500 cpm) were incubated with varying amounts of recombinant SIP-110 in an Sf9 cell supernatant (■) or with the same amount of Sf9 cell supernatant containing an irrelevant control protein (●) for 10 min at 37 °C. [ $^3\text{H}$ ]PtdIns(4,5) $\text{P}_2$  was incubated with 1250 ng of recombinant SIP-110 in an Sf9 cell supernatant (▲) or with the same amount of Sf9 cell supernatant containing an irrelevant control protein (◆) for 30 min at 37 °C. B, the indicated amounts of various recombinant 5-phosphatases in an Sf9 cell supernatant or a similar amount of Sf9 cell supernatant containing an irrelevant control protein was incubated with [ $^{32}\text{P}$ ]PtdIns(3,4,5) $\text{P}_3$  for 15 min at 37 °C.

with detergent lysates of GRB2 and SIP-110 and it was possible this might interfere with an effect of GRB2 on activity, we repeated them using bacterially-expressed GRB2 and SIP-110 from non-detergent cell sonicates. We assayed 0.3 pmol of SIP-110 with increasing amounts of purified bacterial native GRB2. Again there was no effect of GRB2 on hydrolysis of [ $^3\text{H}$ ] $\text{InsP}_4$  by SIP-110 (data not shown). In similar experiments, we also found no effect of GRB2 on SIP-110 hydrolysis of  $\text{PtdIns}(3,4,5)\text{P}_3$  (data not shown). Thus binding of GRB2 to SIP-110 does not affect enzyme activity implying that GRB2 serves mainly as a protein to localize SIP-110 within the cell.

## DISCUSSION

There is a growing family of inositol polyphosphate-5-phosphatases that hydrolyze one or more of the 5-phosphate containing soluble and lipid inositol phosphates. They can be grouped according to their substrate specificity. Group I 5-phosphatases have molecular masses of 32–43 kDa and hydrolyze both  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(1,3,4,5)\text{P}_4$  but neither PtdIns(4,5) $\text{P}_2$  nor PtdIns(3,4,5) $\text{P}_3$ . Originally purified from platelet cytosol as type I 5-phosphatase (35, 46), cDNAs encoding enzymes with similar characteristics have been cloned from a variety of tissue sources (26, 27, 47). Immunoprecipitation of 5-phosphatase activity from human platelets with antisera to the bovine brain and human placental isoforms suggest that these type I activities may be the same enzyme (48, 49).

A second group of 5-phosphatase isoforms hydrolyzes lipid as well as soluble substrates although not all with the same

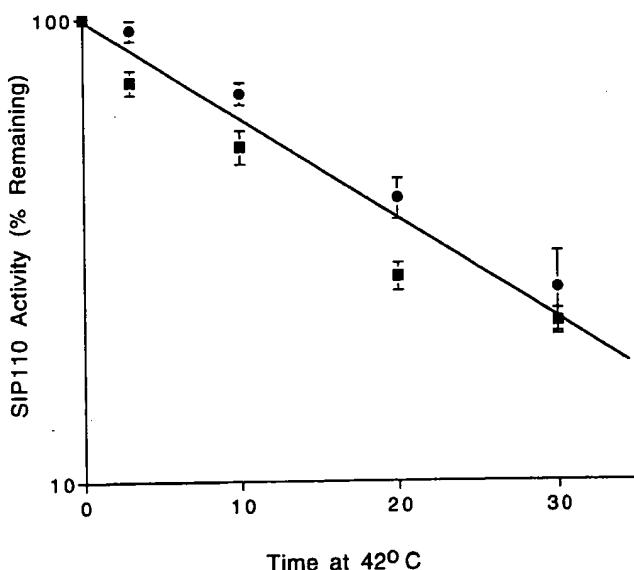


FIG. 5. Kinetics of heat inactivation for the hydrolysis of  $\text{Ins}(1,3,4,5)\text{P}_4$  and  $\text{PtdIns}(3,4,5)\text{P}_3$  by SIP-110. Purified SIP-110 was incubated at 42 °C for the indicated times. The fraction of hydrolyzing activity for  $\text{Ins}(1,3,4,5)\text{P}_4$  (■) and  $\text{PtdIns}(3,4,5)\text{P}_3$  (○) remaining as a function of time was determined. These results are typical of two experiments assayed in duplicate. Error bars indicate S.E.

order of preference. The type II 5-phosphatase was originally isolated as a 75-kDa protein from platelets, although the cDNA could encode larger unprocessed versions of this protein (28, 36, 50). 5-Phosphatase II hydrolyzes  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4,5)\text{P}_4$ ,  $\text{PtdIns}(4,5)\text{P}_2$ , and  $\text{PtdIns}(3,4,5)\text{P}_3$  (36, 44, 51). A second enzyme in this group is the 5-phosphatase disrupted in Lowe syndrome or oculocerebrorenal syndrome (OCRL) (37). This 5-phosphatase has been shown to hydrolyze  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4,5)\text{P}_4$ , and  $\text{PtdIns}(4,5)\text{P}_2$  although it shows a clear preference for the lipid substrate when compared to 5-phosphatase II (29). The ability of OCRL to hydrolyze  $\text{PtdIns}(3,4,5)\text{P}_3$  had not been demonstrated prior to this study. A third 5-phosphatase in this group is synaptojanin that has recently been identified as a 5-phosphatase of 145 kDa that may have an alternatively spliced 170-kDa form (52). This enzyme has an N-terminal region of *SacI* homology and binds to the adaptor protein GRB2. Synaptojanin hydrolyzes  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4,5)\text{P}_4$ , and  $\text{PtdIns}(4,5)\text{P}_2$  (52). Its ability to hydrolyze  $\text{PtdIns}(3,4,5)\text{P}_3$  and its relative preference for soluble *versus* lipid substrates has not been determined (52). In addition to these a number of other 5-phosphatases have been identified in a variety of tissues (53, referenced in Ref. 26), but these have not been sufficiently well characterized to categorize as group I or group II.

A third group of 5-phosphatases is represented by activities that have been identified in association with  $\text{PtdIns}$  3-kinase. These enzymes hydrolyze  $\text{PtdIns}(3,4,5)\text{P}_3$  but not  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4,5)\text{P}_4$ , or  $\text{PtdIns}(4,5)\text{P}_2$  (51, 54).

Group IV 5-phosphatases currently have one member: the 110-kDa SIP-110 (20) characterized in this article and the alternatively spliced 133-kDa SHIP or SIP-130 (20–23). This 5-phosphatase hydrolyzes only 3-phosphate-containing inositol phosphates,  $\text{Ins}(1,3,4,5)\text{P}_4$  and  $\text{PtdIns}(3,4,5)\text{P}_3$ . In addition to the 5-phosphatases described above, there are at least three additional family members based on homologous amino acid sequence derived from cDNA clones: INPPL1 that is closely related to SIP-110 (55) and at least two expressed sequence tags contributed to GenBank.

One of the unusual characteristics of SIP-110 is its ability to associate with GRB2. Experiments reported here show that the binding of SIP-110 to GRB2 has a stoichiometry of approximately

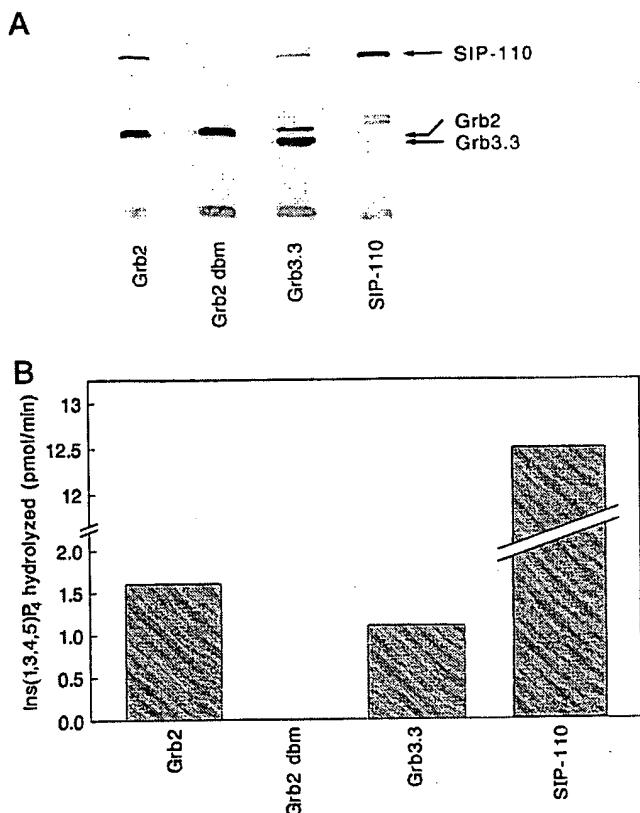


FIG. 6. Association of SIP-110 with GRB2. HA-tagged SIP-110 supernatant was incubated with equimolar amounts of Myc- and HA-tagged native GRB2, GRB2 dbm, or GRB3.3. GRB2 was immunoprecipitated with anti-Myc antiserum and the immunoprecipitate was assayed for association of SIP-110 by Western blotting with anti-HA antiserum (A) or by hydrolysis of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> (B). SIP-110 directly immunoprecipitated with anti-HA antiserum is included as a positive control. The band immediately above GRB2 in all lanes of the immunoprecipitations is IgG. Immunoprecipitations were performed in triplicate and in multiple experiments.

1:1 and confirm that mutations in the SH3 domains of GRB2 can eliminate binding to SIP-110. Another protein related to phosphatidylinositol metabolism also binds to GRB2. The p85 subunit of PtdIns 3-kinase (56) was found to be associated with GRB2 SH3 domains independently of growth factor stimulation. Sos, a guanine nucleotide exchange factor for Ras, also associates with the GRB2 SH3 domains (57–59). GRB2 is proposed to function by bringing its associated proteins into complexes with other tyrosine-phosphorylated proteins such as tyrosine-phosphorylated receptors or tyrosine-phosphorylated Shc (59–61). Studies with GRB2 and Sos suggest that binding of GRB2 to Sos does not affect the guanine nucleotide exchange activity of Sos, and that localizing Sos to the cell membrane by other mechanisms allows for full activation of Ras by Sos (61–63). Our results with SIP-110 suggest a similar role for GRB2. We find that GRB2 binding has no effect on SIP-110 activity *in vitro*, suggesting that GRB2 serves to localize SIP-110 into complexes with other proteins and/or to allow for SIP-110 to associate with the cell membrane. Since one of the two SIP-110 substrates is the inositol lipid,  $\text{PtdIns}(3,4,5)\text{P}_3$ , GRB2 association with a membrane receptor would allow for greater access of SIP-110 to this substrate. While other 5-phosphatases have a higher first-order rate constant for hydrolysis of  $\text{PtdIns}(3,4,5)\text{P}_3$  *in vitro*, localization of SIP-110 in areas of  $\text{PtdIns}(3,4,5)\text{P}_3$  concentration may allow for efficient hydrolysis of this substrate.

Since GRB2 and two other constitutively associated proteins, Sos and the PI 3-kinase, are implicated in regulation of the Ras pathway (39, 59–61, 64), and both SIP-110 substrates have also

been linked to Ras activation (3, 61), we speculate that SIP-110 may have a role in regulating Ras activity. One substrate, Ins(1,3,4,5)P<sub>4</sub>, has been shown to bind a GAP1-like protein and stimulate its Ras GAP activity under some conditions *in vitro* (3). Hydrolysis of Ins(1,3,4,5)P<sub>4</sub> by SIP-110 would decrease this Ras GAP1 activity, resulting in a prolonged activation of Ras. Likewise, a number of studies have suggested that activation of the PI 3-kinase either precedes or follows Ras activation (39, 64). Since SIP-110 hydrolyzes one product of an active PI 3-kinase, PtdIns(3,4,5)P<sub>3</sub>, hydrolysis of this substrate would be predicted to affect the activity of the Ras pathway. Activation of the PI 3-kinase has been shown to activate c-Akt or protein kinase B (18, 19), but there are also several studies showing that members of the protein kinase C family of enzymes are activated by inositol containing phospholipids (65–67). In situations in which PtdIns(3,4,5)P<sub>3</sub> has a positive effect, its hydrolysis by SIP-110 would have negative regulatory consequences. However, recent studies on the phosphorylation of pleckstrin in platelets show that the major phase of thrombin-stimulated phosphorylation is inhibited by the PtdIns 3-kinase inhibitor, wortmannin (68, 69). This phosphorylation may correlate with the production of PtdIns(3,4)P<sub>2</sub> rather than PtdIns(3,4,5)P<sub>3</sub>, and addition of PtdIns(3,4)P<sub>2</sub> in saponin-permeabilized platelets can mimic the effect of thrombin in stimulating pleckstrin phosphorylation (66). Likewise, *in vitro* studies with Akt have demonstrated that PtdIns(3,4)P<sub>2</sub> activates Akt by binding to its pleckstrin homology domain (69). In situations such as this where PtdIns(3,4)P<sub>2</sub> is an activating signal, SIP-110 hydrolysis of PtdIns(3,4,5)P<sub>3</sub> would likewise have an activating effect.

In this study we examine the enzyme activity and products of recombinant 110-kDa SIP-110 and the effects of GRB2 on its activity. This is the newest member of a multigene enzyme family that is likely to influence multiple cell signaling pathways.

**Acknowledgments**—The cDNA encoding 5-phosphatase I was the gift of C. A. Mitchell, Monash Medical School, Victoria 3128, Australia. We thank Xiaoling Zhang (Division of Hematology, Washington University School of Medicine) for baculovirus encoding OCRL, and Anke Klippe (Chiron Corporation) for recombinant PI 3-kinase. We also thank F. Anderson Norris for helpful discussions and useful comments on this work and Cecil B. Buchanan for expert technical assistance.

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